REMARKS

In the Final Office Action dated September 30, 2008, claims 45-46, 49-55, 60-65, 68-71 and 87-91 are pending and under consideration. Claims 45-46, 62-64, 71, and 87-89 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Itskovitz-Eldo et al., Molecular Medicine 6(2): 88-95, February 2000 (hereinafter "Itskovitz-Eldor"). Claims 45-46, 49-55, 60-65, 68-71 and 87-91 are rejected under 35 U.S.C. §103(a) as unpatentable over Itskovits-Eldor in view of Sugi et al., Developmental Dynamics 200: 155-162, 1994 ("Sugi"), Zhu et al. Developmental Dynamics 207: 429-438, 1996 ("Zhu"), Lough et al., Developmental Dynamics 217: 327-342, 2000 ("Lough"), and Klug et al., J Clin Invest 98: 216-224 1996 ("Klug").

This Response addresses each of the Examiner's rejections. Applicant therefore respectfully submits that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claim Amendments

In an effort to advance prosecution, independent claim 45 has been amended to further define the "embryonic cell" as "an endodermal or ectodermal embryonic cell", a feature previously delineated in claim 49. Claim 45 has also been amended to define the "mesodermal cell" as a "cardiomyocyte or vascular endothelial cell". Support for this feature is found throughout the specification, and also found in dependent claims 63 and 64 as previously presented.

Claims 49, 62 and 90 have been canceled without prejudice in view of the amendments to claim 45. Claims 63-64 have been amended to recite "vascular endothelial cell" and "cardiomyocyte", respectively.

Claim 55 has also been canceled without prejudice.

It is respectfully submitted that the foregoing amendments are fully supported by the specification, and do not introduce new matter.

Rejection Under 35 U.S.C. §102(b)

The Examiner maintains that the subject matter of claims 45-46, 62-64, 71 and 87-89 is disclosed by Itskovitz-Eldor. Specifically, the Examiner contends that Itzkovitz-Eldor teaches examination of the differentiation status of cells in the EBs and ES cells grown on mouse embryo fibroblast feeder cells. Itzkovitz-Eldor found robust expression of the ζ-globin mesodermal marker demonstrating that the EBs had begun differentiating. Itzkovitz-Eldor also allegedly found that some significant differentiation occurred in the ES cells grown on feeders, as demonstrated by low levels of expression of the ζ-globin mesodermal marker. Moreover, according to the Examiner, Itzkovitz-Eldor teaches that the differentiating cells acquired characteristic morphologies, distinct for EB regions expressing functional markers, as evident from the appearance of pulsing muscle cells in the EB regions expressing α-cardiac actin (depicted in Figure 4). Thus, the Examiner concludes that the reference teaches that the hES cells are co-cultured with embryonic cells (fibroblasts) to induce differentiation.

In the first instance, Applicant respectfully submits that the claims have been amended to define the "embryonic cell" as "an endodermal or ectodermal embryonic cell". In this regard, the Examiner has admitted in the previous Office Action that Itskovitz-Eldor does not teach that the embryonic cell is an endodermal or ectodermal cell or tissue. See page 5, last sentence of the middle paragraph of the Action dated January 18, 2008. Therefore, the foregoing amendments are sufficient to remove the anticipation rejection based on Itskovitz-Eldor. Notwithstanding, Applicant further respectfully submits the following remarks regarding additional deficiencies of Itskovitz-Eldor.

With respect to the Examiner's position that Itskovitz-Eldor teaches growing ES cells on mouse embryo fibroblasts and allegedly shows significant differentiation among the ES cells, Applicant submits that the alleged significant differentiation among the ES cells is premised solely on an observation of low levels of expression of alpha-fetoprotein (α-FP) (a visceral endoderm marker) and zeta-globin (ζ-globin) (marker for early haematopoietic cells). However, Applicant respectfully submits that there is no showing or disclosure anywhere in Itskovitz-Eldor of cardiac mesoderm or vascular endothelial cells being present among the ES cells at this stage. Specifically, no beating areas were identified in the ES cell culture, and no marker analysis was provided to show that cardiac mesoderm or vascular endothelial cells were present in the ES colonies.

Further, with respect to observations made with EBs, Itskovitz-Eldor discloses that beating cells were identified in the EBs. However, Applicant respectfully submits that these beating cells observed by Itskovitz-Eldor were not produced by co-culturing hES cells with embryonic (endodermal or ectodermal) feeders, as required by the claimed invention. As submitted previously, in Itskovitz-Eldor, the hES cells are co-cultured with embryonic (fibroblast) cells which are used to maintain the hES cells in an undifferentiated state. Once the embryonic fibroblast cells are removed, the undifferentiated hES cells proceed to differentiate to form EBs. See page 89, 2nd column. Differentiation of hES cells only occurred after the hES cells were removed from the co-cultures with embryonic feeders. Thus, Applicant submits that Itskovitz-Eldor does not teach co-culturing the hES cells with embryonic endodermal or ectodermal cells to induce differentiation, as presently claimed.

Moreover, on page 89, second column, Itskovitz-Eldor teaches that the hES cells were cultured in the presence of FGF-2 (bFGF). To induce formation of EBs, hES cells were

transferred into a culture medium which lacked LIF and bFGF (FGF-2) (see first paragraph, second column, page 89). As will be discussed in more detail below (see pages 12-13 of this Response), it is believed that FGF-2 inhibits cardiogenesis and maintains the hES cells in an undifferentiated state. Therefore, the presence of FGF-2 and LIF in the co-culture of hES cells and embryonic feeders, as taught in Itskovitz-Eldor, makes it more probable that differentiation of hES cells into cardiomyocytes had ***not*** occurred.

In view of the foregoing, Applicant respectfully submits that Itskovitz-Eldor does not teach co-culturing of hES cells and embryonic endodermal or ectodermal cells to induce differentiation of the hES cells into mesoderm cells that are cardiomyocytes or vascular endothelial cells, as presently claimed. The rejection under 35 U.S.C. §102(b) based on Itskovitz-Eldo is therefore overcome, and withdrawal thereof is respectfully requested.

Rejection Under 35 U.S.C. §103

The Examiner maintains that Itskovitz-Eldor teaches the use of a co-culture system to induce hES cells to differentiate into a mesodermal cell. The Examiner admits in the previous Office Action that Itskovitz-Eldor does not teach that the embryonic cell used in the co-culture is an endodermal or ectodermal cell or tissue. However, according to the Examiner, Sugi teaches that "anterior endoderm cells regulate the terminal differentiation as opposed to the growth of presumptive cardiac myocytes in mesoderm from the anterior lateral plate." Therefore, the Examiner concludes that Sugi provides sufficient motivation for one of ordinary skill in the art to apply the endodermal embryonic cells of Sugi into the hES cells culture system of Itskovitz-Eldor for inducing differentiation of an undifferentiated hES cell into a mesodermal cell. The additional references to Zhu, Lough and Klug are relied upon by the Examiner in rejecting

certain dependent claims, which include features not disclosed or suggested by the combination of Itskovitz-Eldor and Sugi.

Fundamental Deficiencies of the Primary Reference to Itskovitz-Eldor

The Examiner's obviousness rejection is premised on an interpretation (albeit incorrect) of Itskovitz-Eldor as teaching the use of a co-culture system to induce hES cells to differentiate into a mesodermal cell.

As discussed above, Itskovitz-Eldor fails to teach a co-culture of the hES cells and any embryonic cells (endodermal or ectodermal or otherwise) that induces differentiation of the hES cells into mesoderm cells that are cardiomyocytes or vascular endothelial cells. The co-culture of hES cells with mouse embryonic cells (fibroblasts), taught by Itskovitz-Eldor, was to ensure that the hES cells maintain their undifferentiated state. Even though Itskovitz-Eldor suggests that there was some degree of differentiation in the ES cells, there is absolutely no showing or suggestion anywhere of the presence of cardiomyocytes or vascular endothelial cells in the ES cells, a feature recited in the present claims. Therefore, Itzkovitz-Eldor does not teach the use of a co-culture system to induce the hES cells to differentiate into a cardiomyocyte or vascular endothelial cell.

Further, Applicant respectfully submits that these fundamental deficiencies of Itzkovitz-Eldor are not cured by any of the remaining references. In fact, the remaining references, including Sugi, all relate to the effect of endodermal cells on the terminal differentiation of pre-specified cells (such as mesodermal cells); and none of these remaining references teach how to induce differentiation of undifferentiated, pluripotent hES cells. The deficiencies of these additional references and the impropriety of the Examiner's combination of the references are discussed now in more detail.

Combination of Itskovitz-eldor with Sugi improper.

The Examiner has taken the position that those skilled in the art would have been motivated to apply the endodermal embryonic cells of Sugi into the hES cells culture system of Itskovitz-Eldor for inducing differentiation of an undifferentiated hES cell.

Applicant notes, however, that Itskovitz-Eldor teaches using mouse embryonic fibroblast feeder cells to maintain the cells in an undifferentiated state. The citation teaches removal of the hES cells from the feeder cells in order to induce differentiation and the formation of EBs. From the teaching of Itskovitz-Eldor, the mouse embryonic fibroblast feeder cells clearly had an inhibitory effect on differentiation of hES cells. On the other hand, Sugi teaches that embryonic endoderm or ectoderm cells were instrumental to the cardiogenic differentiation (of a mesoderm cell).

Applicant respectfully submits that the two citations are contrary to each other insofar as the use of an embryonic cell is concerned. Therefore, it would be improper for the Examiner to combine the two references and suggest that one skilled in the art would be motivated to substitute the embryonic fibroblast feeder cells in the co-culture of Itskovitz-Eldor with the embryonic endoderm cells of Sugi in order to arrive at the claimed invention.

Sugi relates to the terminal differentiation process of pre-specified cells.

Sugi teaches that anterior endoderm/mesoderm specifically regulates the terminal differentiation of cardiomyocytes from a pre-specified cell line. This pre-specified cell is not a pluripotent and uncommitted cell type as in the hES cell culture of the claimed invention. Sugi also shows that anterior endoderm cells were able to cause differentiation of Stage 6 mesoderm, which is specified mesoderm that occurs post-gastrulation following ingression through the

primitive streak. The germ layers used from Stage 6 chick embryos are committed embryonic tissue that has undergone specification to either mesoderm, ectoderm or (definitive) endoderm, and as such these cell types are not equivalent to the pluripotent cell types such as undifferentiated hES cells which have no commitment at all. The pre-specified cardiac myocytes of Sugi are already destined to cardiomyocytes and hence will form these cells because they have been pre-specified and require interaction with anterior (definitive) endoderm to complete this specification and terminal differentiation to cardiomyocytes.

Sugi also shows that *only* anterior endoderm cells, but not posterior endoderm cells, were able to cause differentiation of stage 6 mesoderm. This teaches that not all endoderm cells can cause cardiac differentiation of pre-specified cells. More importantly, Sugi further shows that the cardiogenic effects of anterior endoderm appear to be specific to a specific cell population, shown by the inability of posterior endoderm or anterior ectoderm cells to support cardiogenesis. See the abstract on page 155, last seven lines. On page 158, right column, second full paragraph, Sugi discusses "specification" vs. "induction", and expressly states that "the finding that anterior, but not posterior, mesoderm from stage 6 embryos undergoes endoderm-stimulated cardiogenisis suggests that cells in the heart-forming region (HFR) *must* be pre-specified for the cardiogenic lineage." (Emphasis added.)

Applicant respectfully submits that the hES in the present invention are undifferentiated and not pre-specified for the cardiogenic lineage. The Examiner's contention that Sugi provides sufficient motivation for one to apply the endoderm cells to hES cells is completely unsupported, and in fact, contrary to Sugi's explicit teaching that the cardiogenic effects of anterior endoderm were specific to pre-specified cells. Based on Sugi's disclosure, those skilled in the art would not have had any expectation of success that such undifferentiated

hES cells, which do not have a pre-specification, would proceed down the cardiogenic path when co-cultured with endoderm cells.

Itskovitz-Eldor relates to human ES cells and Sugi relates to chickens

Applicant further submits that Sugi is not relevant to the claimed invention also because Sugi relates to chickens. The present claims are directed to differentiating human ES cells. Even among mammalian species, mouse ES cells have been shown to behave differently from human embryonic stem cells. For instance, LIF is essentially to culturing mouse ES cells, but is not required in culturing human ES cells. Therefore, those skilled in the art would have had no reasonable expectation of success in applying Sugi's teaching, which relates to avian cells, to human ES cells.

Combination of references also improper in light of Zhu's teaching of FGF2

The Examiner admits that the combination of Itskovitz-Eldor and Sugi does not teach that the embryonic cell is derived from visceral endoderm tissue or visceral endoderm-like tissue, or from an early post-gastrulation embryo, or an embryonic cell line. The Examiner has relied on Zhu and Lough in an attempt to cure such deficiencies. The Examiner has further relied on to supply the teaching of a genetically modified hES cell, apparent missing in the other references.

Initially, Applicant observes that Zhu and Lough both relate to the terminal differentiation of mesoderm cells to cardiomyocytes. Therefore, similar to Sugi, these citations are not relevant to the differentiation of hES cells or of any other unspecified undifferentiated cells. Zhu and Lough provide no motivation, and certainly no reasonable expectation of success in inducing differentiation of undifferentiated, pluripotent hES cells.

Further, Applicant respectfully submits that the disclosure of Zhu would have rendered inoperable or at least improper, the combination of references including the combination of Itskovitz-Eldor and Sugi.

Specifically, Zhu teaches that FGFs (Fibroblast growth factors) are potentially one such secreted factor produced by the anterior (definitive) endoderm that is involved in the terminal differentiation of cardiomyocytes in the embryo. FGF antigens were detected only in late stage embryos. For example, FGF2 (and FGF 1 and 4 which occur later) were exclusively detected in Stage 5+ endoderm and later in the myocardium. This endoderm is of definitive embryonic endoderm characteristics (e.g. notochord), *not* extraembryonic visceral endoderm (i.e. the hypoblast in the chick embryo). The loss of expression of these factors also paralleled the loss of proliferation of the myocardium and its terminal differentiation to cardiomyocytes, suggesting a major role for FGF in the proliferation of cardiomyocytes at this stage of embryogenesis provided by this definitive endoderm.

On the other hand, FGF2 was known to play a role in maintaining the pluripotent state of hES cells at the time. FGF2 was (and still is) an essential media supplement to hES cell media. The critical role of FGF2 (bFGF) in maintaining hES cell pluripotency is documented by "Basic fibroblast growth factor support of human embryonic stem cell self-renewal.," Levenstein et al., Stem Cells, 24(3):568-74 (2006); and "Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells, " Xu et al., Nat Methods,2(3):185-90 (2005). Copies of these articles are provided as **Exhibits 1-2.**

Applicant also refers to the Itskovitz-Eldor reference, in which LIF and FGF2 were *withdrawn* from the hES cell culture media to allow the EBs to form and subsequent

cardiogenesis to occur. This would indicate that an FGF (particularly FGF2 that is removed from the media) is inhibitory to the formation of cardiomyocytes from undifferentiated hES cells.

Therefore, in light of the disclosure of Zhu that FGF2 was produced by an anterior definitive endoderm, and given the knowledge in the art that FGF2 was used as a media component to maintain hES cell pluripotency (including the Itskovitz-Eldor reference), those skilled in the art would not have combined Zhu with the other cited references; and in fact, those skilled in the art would not have even combined Itskovitz-Eldor with Sugi to apply the anterior definitive endoderm of Sugi (which would secrete FGF2, according to Zhu) in an attempt to influence differentiation of hES cells in the culture of Itskovitz-Eldor.

Summary

It is respectfully submitted that Itzkovitz-Eldor as a primary reference fails to teach co-culturing hES cells with embryonic cells to induce differentiation of hES cells into cardiomyocyte or vascular endothelial cells. The combination of Itzkovitz-Eldor and Sugi is improper, because the use of embryonic cells taught by the two references are contrary to each other, and because the cardiogenic effects of anterior endoderm cells, disclosed by Sugi, are expressly taught to be limited to pre-specified cells. The combination of Itzkovitz-Eldor and Sugi is even further compromised in light of Zhu, showing FGF2 being secreted by anterior endoderm, a factor known for use in maintaining the pluripotency of hES cells. Hence, those skilled in the art would not have had motivation to combine Itzkovitz-Eldor and Sugi, and clearly would not have had a reasonable expectation of success in arriving at the claimed invention.

Therefore, Applicant respectfully submits that the presently claimed invention, as a whole, is not obvious over the cited references. Withdrawal of the rejection under 35 U.S.C. §103(a) is respectfully requested.

Conclusion

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

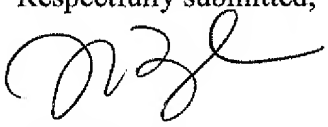

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EXHIBIT 1

STEM CELLS
EMBRYONIC STEM CELLS

Basic Fibroblast Growth Factor Support of Human Embryonic Stem Cell Self-Renewal

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Key Words. Human embryonic stem cell • Fibroblast growth factor

ABSTRACT

Human embryonic stem (ES) cells have most commonly been cultured in the presence of basic fibroblast growth factor (FGF2) either on fibroblast feeder layers or in fibroblast-conditioned medium. It has recently been reported that elevated concentrations of FGF2 permit the culture of human ES cells in the absence of fibroblasts or fibroblast-conditioned medium. Herein we compare the ability of unconditioned medium (UM) supplemented with 4, 24, 40, 80, 100, and 250 ng/ml FGF2 to sustain low-density human ES cell cultures through multiple passages. In these stringent culture conditions, 4, 24, and 40 ng/ml FGF2 failed to sustain human ES cells through three passages, but 100 ng/ml sustained human ES cells with an effectiveness comparable to conditioned medium (CM). Two human ES cell lines (H1 and H9) were maintained for up to 164 population doublings (7 and 4 months) in UM supplemented with 100 ng/ml FGF2. After prolonged culture, the cells formed teratomas when injected into severe combined immunodeficient beige mice and expressed markers characteristic of undifferentiated human ES cells. We also demonstrate that FGF2 is degraded more rapidly in UM than in CM, partly explaining the need for higher concentrations of FGF2 in UM. These results further facilitate the large-scale, routine culture of human ES cells and suggest that fibroblasts and fibroblast-conditioned medium sustain human ES cells in part by stabilizing FGF signaling above a critical threshold.
STEM CELLS *2006;24:568–574*

INTRODUCTION

Embryonic stem (ES) cells can be expanded indefinitely while maintaining the potential to form any cell type of the body [1–4]. Both human and mouse ES cells were initially isolated on fibroblast feeder layers in medium containing serum; however, the growth factors that maintain human and mouse ES cells are distinct. In the presence of serum and leukemia inhibitory factor (LIF), the resulting activation of the JAK/STAT3 pathway supports feeder-independent growth of mouse ES cells [5, 6]. In comparable culture conditions, LIF does not maintain human ES cells, and the JAK/STAT3 pathway does not appear to become activated in conditions that maintain human ES cells [4]. In serum-free conditions, combined BMP4 and LIF activities are sufficient to support the clonal growth of mouse ES cells [7]. However, when BMP4 is added to human ES cells in culture conditions that would otherwise support undifferentiated proliferation, rapid differentiation occurs [8].

In contrast to mouse ES cells, fibroblast growth factor (FGF) signaling appears to be of central importance to human ES cell self-renewal. FGF2 (4 ng/ml) and a commercially available serum substitute support human ES cell growth on fibroblasts [9] or in fibroblast-conditioned medium [10]. It has recently been shown that elevated levels of FGF2 can sustain human ES cells through long-term cultures in the absence of fibroblasts [11–14]. Although 40 ng/ml FGF2 can sustain high-density human ES cell cultures through multiple passages in the absence of fibroblasts, there is significantly more background differentiation compared with control cells cultured in conditioned medium (CM) [11] (Fig. 1C vs. 1A).

FGF proteins play a role in diverse developmental pathways, and antagonism between FGF and bone morphogenetic protein (BMP) signaling is a repeated theme [15, 16]. We have previously shown that the level of BMP activity in unconditioned medium (UM) is sufficient to cause human ES cell differentiation and that conditioning the medium reduces or blocks BMP activity [11]. UM supplemented with 40 ng/ml FGF2 and the BMP antagonist noggin sustained feeder-free, undifferentiated human ES cell proliferation through prolonged culture, with

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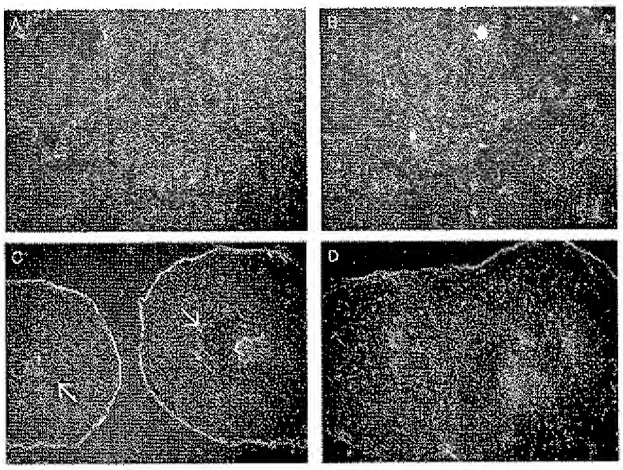


Figure 1. Morphology of human embryonic stem cells grown in high FGF2 concentrations. 5× phase contrast images show representative morphology of H14 cells maintained for 5 days in CM **(A)**, UM **(B)**, UM 40 **(C)**, or UM 100 **(D)**. Arrows identify centralized regions of differentiation within UM 40 colonies. Abbreviations: CM, conditioned medium plus 4 ng/ml FGF2; UM, unconditioned medium plus 4 ng/ml FGF2; UM 40, unconditioned medium plus 40 ng/ml FGF2; UM 100, unconditioned medium plus 100 ng/ml FGF2.

background differentiation levels comparable to those observed in CM [11]. Furthermore, elevating FGF2 levels to 100 ng/ml in UM suppressed BMP signaling to a level comparable to that achieved either by the addition of noggin or by fibroblast-conditioning the medium. Indeed, at 100 ng/ml FGF2, background differentiation was minimal, and supplementation with noggin conferred no additional benefit in short-term human ES cell cultures (data not shown).

Herein we compare growth curve analyses of low-density human ES cell cultures through multiple passages in UM supplemented with 4, 24, 40, 80, 100, and 250 ng/ml FGF2. We find that culture in UM plus 100 ng/ml FGF2 closely parallels the proliferation and low background differentiation rate obtained using CM and supports long-term, undifferentiated, feeder-independent culture of human ES cells. We further demonstrate that FGF2 is more rapidly degraded in UM than in CM, so that ES cells are exposed to dramatically different levels of FGF2 in UM at the beginning and end of the 24 hours between routine media changes. This suggests that the maintenance of FGF2 levels above a critical threshold throughout the culture period is essential and that one of the effects of culturing on fibroblasts or in CM is stabilization of the FGF signal.

Materials and Methods

Cell Lines and Cell Culture

The human ES cell lines H1, H7, H9, and H14 were cultured on Matrigel (BD Biosciences, Franklin Lakes, NJ, http://www.bdbioscience.com) in CM as previously described [10]. UM contained 80% Dulbecco's modified Eagle's medium/F-12 and 20% knockout serum replacement supplemented with 1 mM L-glutamine, 1% nonessential amino acids (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), and 4 ng/ml human FGF2 (Invitrogen). UM 24, 40, 80, 100, and 250 contained UM supplemented with 24, 40, 80, 100, and 250 ng/ml FGF2, respectively. UM 4/retinoic acid (RA) contained UM supplemented with 4 ng/ml human FGF2 and 10 μM retinoic acid (Sigma-Aldrich). Cultures of human ES cells were routinely passaged onto Matrigel-coated plates in clumps at approximately weekly intervals by exposure to dispase (1 mg/ml; Invitrogen). Cells were transitioned into test media either by direct passage from CM or by directly thawing from frozen stock.

Immunofluorescence

H1 cells growing in CM (22 passages) and UM 100 (24 passages) and H9 cells growing in UM 100 (10 passages) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, http://www.emsdiasum.com) for 15 minutes at room temperature. Cells were then washed three times for 5 minutes in 150 mM NaCl, 40 mM K_2HPO_4, and 10 mM KH_2PO_4. After blocking in 5% milk, cells were probed with either an anti-human, octamer-binding transcription factor-3/4 (OCT4) mouse monoclonal or an isotype control antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com/). Cells were washed and then probed with an Alexa Fluor anti-mouse secondary antibody (Invitrogen) for 1 hour in the dark at room temperature. Finally, fluorescent images were captured with a DM IRB microscope (Leica, Frankfurt, Germany, http://www.leica.com) and analyzed with IP Lab software (Scanalytics Incorporated, Fairfax, VA, http://www.scanalytics.com).

Western Blotting

H1 cells grown in CM or UM 100 (passage 24 or 26, respectively) were individualized for 10 minutes at 37°C with Trypsin/EDTA (Invitrogen), counted with a hemocytometer, and resuspended in 8 M urea/100 mM Tris (pH 7.2). Lysates from 1.5×10^6 cells were run on 4%–20% gradient polyacrylamide gels along with biotinylated standards and transferred to nitrocellulose (all from Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com). Filters were blocked for 1 hour at room temperature in 1× blocking buffer (Sigma-Aldrich) in phosphate-buffered saline (PBS). Anti-human OCT4 or NANOG primary antibodies (Santa Cruz Biotechnology, Inc.; R&D Systems, Minneapolis, http://www.rndsystems.com) were diluted 1:500 or 1:100 in 1× blocking buffer and probed for 1 hour at room temperature. Filters were washed 2 × 5 minutes in Tris-buffered saline/Tween 20 (TBST; 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, and 0.05% Tween-20). Dilutions (1:2000) of appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) along with 1:5000 dilutions of avidin-HRP (Bio-Rad Laboratories) in 1× blocking buffer were added and probed for 1 hour at room temperature. Filters were washed 2 × 5 minutes in TBST, and signals were detected by chemiluminescence with enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, U.K., http://www.gehealthcare.com).

Reverse Transcription-Polymerase Chain Reaction and Fluorescence-Activated Cell Sorting Analysis

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from H1 cells cultured in CM or UM 100 (24 or 26 passages, respectively) using an RNeasy Mini Kit

(Qiagen, Valencia, CA, http://www1.qiagen.com) following the manufacturer's recommendations. Transcripts were assayed from 1 μg of total RNA with 35 rounds of PCR amplification using OneStep RT-PCR Kit (Qiagen) with previously described gene-specific primers [11] and visualized on ethidium bromide-stained agarose gels.

For fluorescence-activated cell sorting (FACS) analysis, cells were removed from the culture dish with Trypsin/EDTA (Invitrogen) containing 2% chick serum (ICN, Costa Mesa, CA, http://valeant.com) for 10 minutes at 37°C and resuspended in FACS buffer (PBS + 2% fetal bovine serum + 0.1% sodium azide). The cells (probed for internal markers) were fixed with 0.1% paraformaldehyde for 10 minutes at 37°C and then permeabilized with 90% methanol (Fisher Scientific, Rochester, NY, https://www1.fishersci.com) for 30 minutes on ice. 1 to 5 × 10^5-treated (OCT4) or nontreated (stage-specific embryonic antigen-4 [SSEA4] or tumor rejection antigen 1-60 [Tra1–60]) cells were then probed for 2 hours at room temperature with a 1:100 dilution of the specific monoclonal antibody or an appropriate isotype control antibody (Santa Cruz Biotechnology, Inc.) in FACS buffer (+0.1% Triton-X100 for OCT4). Cells were then washed and probed in FACS buffer (+0.1% Triton-X100 for OCT4) with 1:1000 dilution of an Alexa Fluor anti-mouse secondary antibody (Invitrogen) for 1 hour in the dark at room temperature. The Triton-X100 was washed away, and the cells were sorted using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences).

FGF2 Stability Assays

CM and UM samples were prepared and supplemented with 4, 24, 40, 80, 100, or 250 ng/ml FGF2. Two milliliters of each sample were placed at 37°C in a single well of day 3 (H1) ES cells cultured in CM + 4 ng/ml FGF2 in a six-well tissue culture dish (Nunc, Rochester, NY, http://www.nuncbrand.com), and the remainder was stored at 4°C. After overnight incubation, FGF2 concentrations were determined in 96-well plates (Nunc) using a Human FGF-basic ELISA Development Kit following the manufacturer's recommendations (PeproTech, Rocky Hill, NJ, http://www.peprotech.com). Measurements were performed on a Tecan GENios Pro plate reader (Tecan US, Research Triangle Park, NC, http://www.tecan-us.com) and analyzed with Magellan5 software (Tecan US). FGF2 concentrations following incubation at 4° or 37°C were compared using student's t test.

Results

High Concentrations of FGF2 Support Undifferentiated Human ES Cell Growth

H14 cells (passage 36) grown in CM (4 ng/ml FGF2) displayed the standard morphology of pluripotent human ES cells (small cell sizes, large nuclear/cytoplasmic ratio, defined colony borders; Fig. 1A). In contrast, cells grown in UM (4 ng/ml FGF2) showed significantly differentiated morphologies (flattened, "cobblestone-like" cells; Fig. 1B). Confirming previous results [11, 13], we found that UM supplemented with 40 ng/ml FGF2 (UM 40) supported human ES cells through multiple passages, but a central region of differentiation consistently formed within ES cell colonies (Fig. 1C, arrowheads). However, UM supplemented with 100 ng/ml FGF2 (UM 100) prevented this central differentiation (Fig. 1D; Fig. 3A, i, ii).

Dose Response for FGF2 Support of Human ES Cells

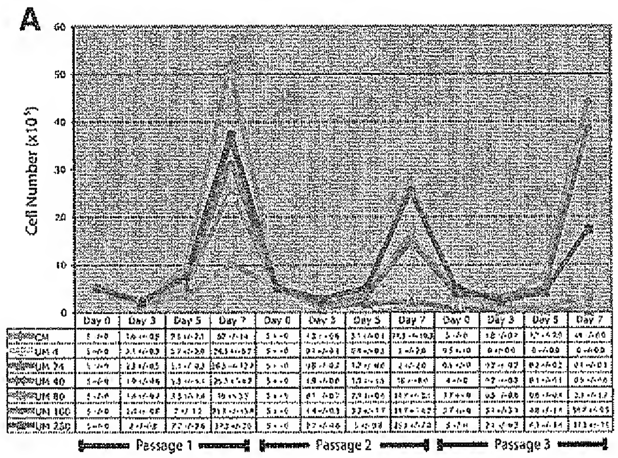
To examine the attachment efficiency and proliferation rates of human ES cells cultured in FGF2-supplemented UM, H9 cells (passage 50/10 in CM) were grown in CM, UM 4, UM 24, UM 40, UM 80, UM 100, or UM 250. 5 × 10^5 cells were plated in triplicate culture wells, and cell numbers were counted on days 3, 5, and 7 after plating. On day 7, cells were analyzed by FACS for OCT4, SSEA4, and Tra1-60 expression. After 7-day periods, if sufficient cells proliferated in a particular condition, 5 × 10^5 cells were replated for a total of three passages. UM 4, UM 24, and UM 40 all failed to support H9 culture through these three passages of low-density culture. CM, UM 100, UM 250, and with somewhat less effectiveness, UM 80, were all capable of sustaining undifferentiated human ES cell proliferation through three passages (Fig. 2). Both the percentage of cells that expressed markers of pluripotency and the total number of cells after three passages in UM 100 were comparable to cells cultured in CM (Fig. 2). In total, comparable FGF2 dose-response results were compiled using three separate ES cell lines as well as alternate lots of both knockout-serum replacement and Matrigel, highlighting the central role of FGF2 signaling in human ES cell self-renewal.

Long-Term Culture of Low-Passage Human ES Cells in 100 ng/ml FGF2

Independent human ES cell lines H9 and H1 were maintained in UM 100 for 16 and 33 passages, respectively, or for a period of 4–7 months. H1 cells (passage 41) had previously been cultured in CM for two passages, whereas H9 cells (passage 22) were thawed directly into UM 100 from frozen stocks previously grown only on fibroblasts. The karyotypes of the H1 cells were examined after 10 passages in UM 100. The karyotypes of H9 cells were examined after 17 passages in UM 100. Both were confirmed normal (data not shown).

Human ES cell lines H1 (24 passages in UM 100) and H9 (10 passages in UM 100) were analyzed for OCT4 expression by immunocytochemistry, and both demonstrated staining for OCT4 that was comparable to CM-cultured human ES cells (Fig. 3A, iv and v vs. vi). RT-PCR was performed on H1 cells (24 passages in CM or 26 passages in UM 100), confirming the continued expression of human ES cell markers OCT4, NANOG, ZFP42 (hRex1), and telomerase reverse transcriptase (TERT) (Fig. 3B). Whole-cell protein extracts from the same H1 cells were also analyzed for both OCT4 and NANOG protein expression by Western blot. Again, UM 100 supported expression levels comparable to CM controls (Fig. 3C). Finally, H1 (passage 30) cells were analyzed by FACS for internal (Oct4) and cell surface (SSEA4, Tra1–60) markers after 7 days of culture in CM, UM 100, or UM 4/retinoic acid. Both CM- and UM 100-cultured ES cells highly expressed pluripotency markers, whereas ES cells cultured in suboptimal growth conditions down-regulated these genes (Fig. 3D, i–vi vs. vii–ix).

To test whether UM 100-cultured human ES cells maintain developmental potential, three severe combined immunodefi-



B FACS Analysis (Percent Positive)

	Oct4	SSEA4	Tra1-60
CM	98	95	87
UM 250	97	95	88
UM 100	97	96	90
UM 80	97	96	90
UM 40	Insufficient Cell Numbers		
UM 24	Insufficient Cell Numbers		
UM 4	Insufficient Cell Numbers		

Figure 2. FGF2 dose response for human embryonic stem cell self-renewal. **(A):** Growth curve analysis of H9 cells cultured in CM, UM 4, UM 24, UM 40, UM 80, UM 100, and UM 250 for three passages. 5 × 10^5 cells from CM-cultured H9 cells were plated on day 0 of passage 1. Cell numbers were counted from triplicate wells on days 3, 5, and 7 of each passage. Initial plating density and sampling times were repeated, when possible, for three passages. Average cell numbers (×10^5 cells) with standard deviations are listed below the graph for each condition and time point. **(B):** For conditions that allowed cell survival through three passages, cells were analyzed on day 7 of passage 3 by fluorescence-activated cell sorting for multiple human embryonic stem cell markers. Abbreviations: CM, conditioned medium plus 4 ng/ml FGF2; UM 4, unconditioned medium plus 4 ng/ml FGF2; UM 24, unconditioned medium plus 24 ng/ml FGF2; UM 40, unconditioned medium plus 40 ng/ml FGF2; UM 80, unconditioned medium plus 80 ng/ml FGF2; UM 100, unconditioned medium plus 100 ng/ml FGF2; UM 250, unconditioned medium plus 250 ng/ml FGF2; OCT4, octamer-binding transcription factor-3/4; SSEA4, stage-specific embryonic antigen-4; Tra1-60, tumor rejection antigen 1-60.

cient beige mice were each injected with 8 × 10^6 H1 cells (12 passages in UM 100). Each animal formed a teratoma that exhibited advanced differentiation of multiple lineages (Fig 4). Therefore, we conclude that UM 100 supports long-term culture of human ES cells with an effectiveness comparable to CM.

FGF2 Stability

One hundred nanograms per milliliter (5.8 nM) FGF2 is substantially above both the published high-affinity K_d values for FGF receptors (typically in the range of 20–200 pM) [17] as well as the 4 ng/ml FGF2 (230 pM) used to support human ES cells in CM. However, FGF proteins that are not bound to heparin/heparan sulfates are known to be sensitive to proteolysis and thermal denaturation [18]. If FGF2 is unstable in our current culture conditions, then the requirement for this high level of initial FGF2 could be explained by degradation over the 24 hours between medium changes to a level below an important signaling threshold.

We performed enzyme-linked immunosorbent assay (ELISA)-based assays to examine the concentrations of FGF2 after routine culture. No substantial loss of FGF2 was detected in either CM- or UM-based media after overnight incubation at 4°C (Fig. 4). However, upon overnight incubation at 37°C, we found a significant reduction of FGF2 levels regardless of the initial concentration (4, 24, 40, 80, 100, or 250 ng/ml; $p < .01$ for all concentrations). In addition, consistently lower FGF2 levels were detected in UM cultures than were detected in CM culture following incubation (Fig. 4). Thus, it appears that one function of the conditioning process is FGF2 stabilization and that high initial concentrations of FGF2 can compensate for its instability in UM. However, there were considerable levels of FGF2 remaining in UM 40 after 24 hours of culture on H1 cells, so differences in FGF2 stability alone are likely not sufficient to explain the differences in culture performance observed between CM, UM 40, and UM 100.

Large-Scale Human ES Cell Culture

Eliminating the need for fibroblast production greatly reduces the labor required for large-scale expansion of human ES cells. To demonstrate the effectiveness of large-scale culture with UM 100, we began with a single 6-well tissue culture dish of H1 cells and subsequently expanded to 40 × 100 mm culture dishes over 3 passages (26 days total). This expansion resulted in 1.8 × 10^9 ES cells, 92% of which were OCT4 positive (data not shown).

Discussion

Fibroblasts secrete multiple growth factors, including FGFs, transforming growth factor β (TGFβ), activin, Wnts, and antagonists of BMP signaling. Media incorporating different combinations of these factors have been reported to sustain human ES cells in the absence of fibroblasts [11–14, 19–22]. In each of these feeder-free media formulations, an FGF family member has been present. Because FGF2 stability is enhanced in CM (Fig 4), fibroblasts likely secrete either protease inhibitors or binding proteins that modulate FGF2 stability. Heparin and heparan sulfate proteoglycans modulate the stability and activity of FGFs by forming high-affinity binding complexes [23]. However, at high concentrations FGF proteins can signal through direct interaction with FGF receptors, and the requirement for these complexes might be circumvented [24]. Thus, the addition of appropriate heparan sulfate proteoglycans to human ES cell medium may further improve the culture of human ES cells and reduce the required FGF2 concentrations. To date, we have been unsuccessful in using either exogenous heparin or heparan sulfate proteoglycans to reduce the required concentrations of FGF2 (data not shown). Because a large variety of heparin and heparan sulfates exist in multiple modified states, it will be important to identify which, if any, are secreted by fibroblast feeder cells.

FGF signaling is dose-dependent. It has been reported that, in some instances, thresholds must be maintained for a minimum of 12 hours for appropriate signaling [25]. Whereas high-affinity binding constants for FGF2 are in the picomolar range,

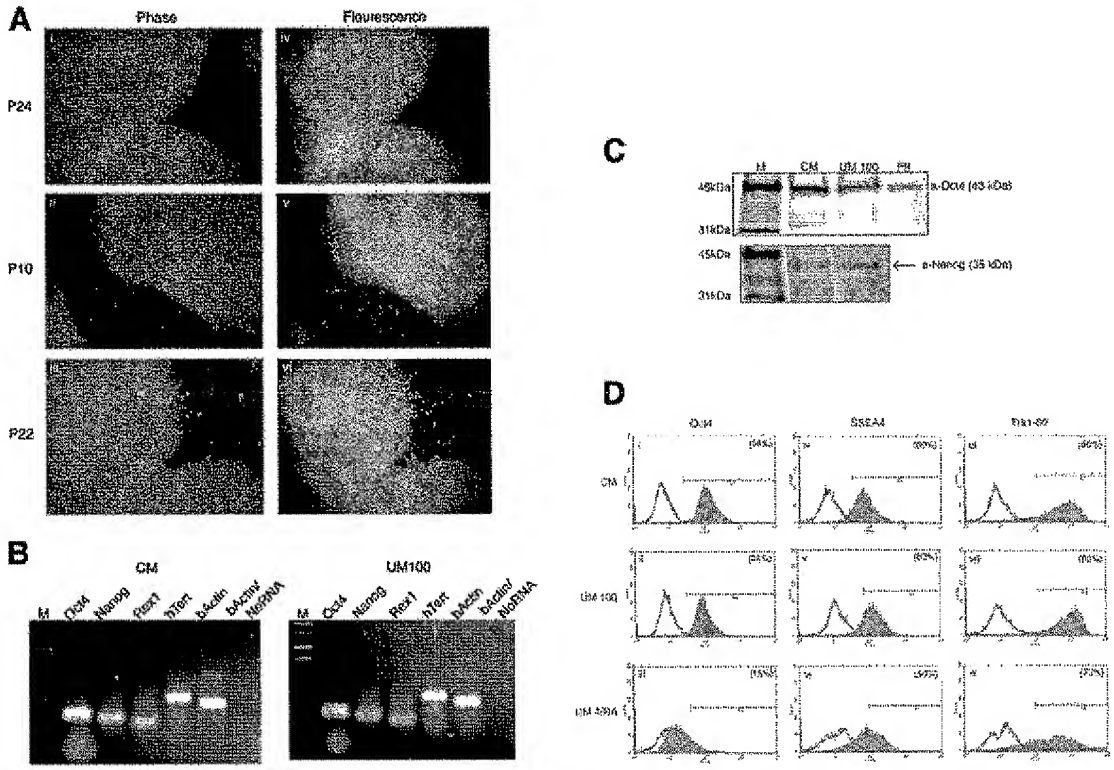


Figure 3. Human embryonic stem cell marker expression in UM 100-cultured cells. **(A):** 5× phase contrast and immunofluorescence for OCT4 on H1 cells (i, iv: 24 passages in UM 100), H9 cells (ii, v: 10 passages in UM 100), and H1 CM-cultured controls (iii, vi: 23 passages). **(B):** Reverse transcription-polymerase chain reaction analysis for molecular markers expressed in H1 cells cultured in CM (17 passages) or UM 100 (19 passages). **(C):** Western blot analysis for OCT4 and NANOG of H1 cells cultured in CM (24 passages) or UM 100 (26 passages). F9 cell lysate (Santa Cruz Biotechnology, Inc.) served as an OCT4-positive control. **(D):** Fluorescence-activated cell sorting analysis of OCT4 (i–iii), SSEA4 (iv–vi), and Tra1-60 (vii–ix) expression by H1 cells (passage 30) cultured 7 days in CM (i, iv, vii), UM 100 (ii, v, viii), or UM 4/RA (iii, vi, ix). Abbreviations: CM, conditioned medium plus 4 ng/ml FGF2; UM 100, unconditioned medium plus 100 ng/ml FGF2; M, markers; OCT4, octamer-binding transcription factor-3/4; Rex1, Reduced expression-1; hTERT, human telomerase reverse transcriptase; F9, mouse embryonal carcinoma cells; SSEA4, stage-specific embryonic antigen-4; Tra1-60, tumor rejection antigen 1-60; UM 4/RA, unconditioned media plus 4 ng/ml FGF2 and 10 μM retinoic acid.

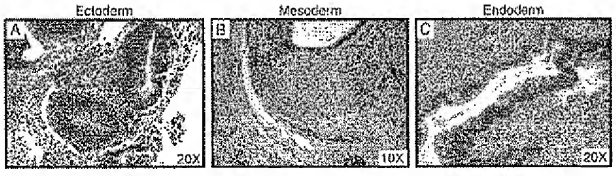


Figure 4. Teratoma formation. UM 100-cultured H1 cells form teratoma-generating structures representative of all developmental lineages. **(A):** Neural rosette. **(B):** Cartilage. **(C):** Epithelium. Abbreviation: UM 100, unconditioned medium plus 100 ng/ml FGF2.

nanomolar levels are reported for low-affinity binding [17]. We find that overnight incubation at 37°C results in a significant reduction of FGF2 regardless of the initial concentration (Fig. 5). However, our data show that in UM 100 FGF2 concentrations remain in the nanomolar range (1.8 nM) throughout the culture period, whereas FGF2 levels in media with lower starting concentrations do not (Fig. 5). Interestingly, our data show that FGF2 levels in UM 80 straddle the nanomolar benchmark (970 pM–1.1 nM), perhaps explaining the capacity of this media formulation to sustain ES cells for three passages in our low-density growth curve assay, albeit less effectively than UM 100 (Figs. 2 and 5).

Limiting spontaneous differentiation is essential for maintaining long-term human ES cell culture. BMP signaling increases differentiation in human ES cells, and BMP antagonists such as noggin have a positive effect on human ES cell culture [11]. Our recent study showed that FGF2 itself suppresses BMP signaling in human ES cells [11]. However, our finding that FGF2 levels in UM degrade significantly during a 24-hour culture period suggests that cells cultured in UM with FGF2 concentrations below 80 ng/ml can be exposed to significant periods of suboptimal FGF signaling, allowing them to respond to differentiation-inducing signals, including BMPs. At higher initial FGF2 concentrations (e.g., 100 ng/ml), where adequate FGF concentrations are maintained throughout the culture period, additional antagonism of the BMP pathway no longer appears beneficial. It is currently unclear whether other factors observed to have beneficial effects at lower FGF2 concentrations (such as TGFβ, activin, or Wnts) will further improve culture at these higher FGF2 concentrations, but UM 100 is already an effective medium for propagating human ES cells.

UM 100 addresses several broad goals for human ES cell media improvement. The first is to simplify human ES cell culture so that laboratories with little or no previous ES cell

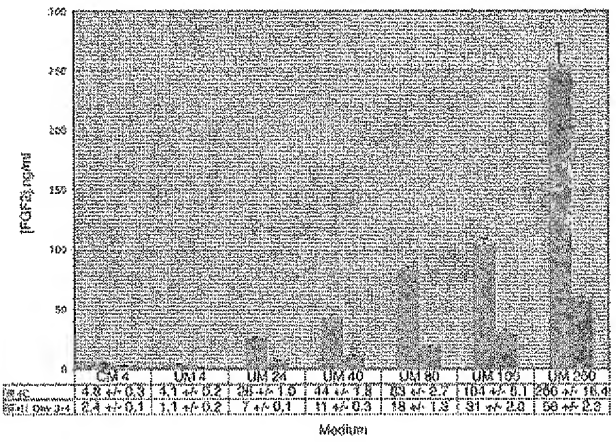


Figure 5. FGF2 stability. Different starting concentrations of FGF2 added to conditioned or unconditioned medium were analyzed for remaining FGF2 concentrations after overnight incubation at 4°C (blue bars) or on H1 cells at 37°C (red bars). Samples were subjected to enzyme-linked immunosorbent-based assays for final FGF2 concentrations. Values with standard deviations are listed below bars in nanograms per milliliter. Abbreviations: CM 4, conditioned medium plus 4 ng/ml FGF2; UM 4, unconditioned medium plus 4 ng/ml FGF2; UM 24, unconditioned medium plus 24 ng/ml FGF2; UM 40, unconditioned medium plus 40 ng/ml FGF2; UM 80, unconditioned medium plus 80 ng/ml FGF2; UM 100, unconditioned medium plus 100 ng/ml FGF2; UM 250, unconditioned medium plus 250 ng/ml FGF2.

experience can reliably produce cells in numbers useful in a research setting. UM 100 greatly simplifies the propagation of human ES cells by removing variation and technical hurdles associated with fibroblast feeder cells. As a result, cell numbers greater than 10^9 can be readily cultured within three passages. Second, more defined culture conditions will allow a careful dissection of the signaling pathways essential for human ES cell self-renewal. UM 100 clearly highlights the importance of FGF signaling. However, UM 100 is not yet a defined culture system, as both the cell matrix (Matrigel; BD Biosciences) and serum replacement (Invitrogen) contain complex, poorly defined mixtures of proteins and other molecules that influence self-renewal and differentiation. And finally, improved culture conditions will facilitate the clinical use of human ES cell-based therapies. Clearly, challenges remain for this goal, as the matrix and serum replacement contain animal products and provide a possible route for introduction of pathogens to the culture system. Nonetheless, UM 100 is a simple, efficient, feeder-independent culture system that sheds additional light on the mechanism of FGF2 support of human ES cell proliferation.

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DISCLOSURES

J.T. owns stock in and has served on the Board of Cellular Dynamics International (Madison, WI) within the last 2 years.

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Basic Fibroblast Growth Factor Support of Human Embryonic Stem Cell Self-Renewal

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EXHIBIT 2

Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells

Ren-He Xu[1,2], Ruthann M Peck[1], Dong S Li[1], Xuezhu Feng[2], Tenneille Ludwig[2] & James A Thomson[1,2]

Human embryonic stem cells (hESCs) are routinely cultured on fibroblast feeder layers or in fibroblast-conditioned medium (CM). Bone morphogenetic proteins (BMPs) have previously been shown to induce hESC differentiation, in apparent contrast to mouse embryonic stem (ES) cells, in which BMP4 synergizes with leukemia inhibitory factor (LIF) to maintain self-renewal. Here we demonstrate that hESCs cultured in unconditioned medium (UM) are subjected to high levels of BMP signaling activity, which is reduced in CM. The BMP antagonist noggin synergizes with basic fibroblast growth factor (bFGF) to repress BMP signaling and sustain undifferentiated proliferation of hESCs in the absence of fibroblasts or CM. These findings suggest a basic difference in the self-renewal mechanism between mouse and human ES cells and simplify the culture of hESCs.

hESCs were initially isolated on fibroblast feeder layers in medium containing serum[1,2], conditions remarkably similar to those first used to isolate mouse ES cells[3,4]. Yet despite these initial culture similarities, the factors that mediate self-renewal of mouse and human ES cells appear to be distinct. LIF is used to culture mouse ES cells[5,6]. LIF binds a heterodimer of the LIF receptor and gp130 that activates JAK/Stat3 signaling, and activated Stat3 is sufficient to sustain undifferentiated proliferation of mouse ES cells cultured in serum. It has recently been reported that, in the absence of serum, BMPs can synergize with LIF to maintain self-renewal of mouse ES cells by inducing expression of *Id* genes[7]. Addition of LIF to culture medium[1] or activation of Stat3 (ref. 8) does not sustain hESCs in conditions that would support mouse ES cells. Of all the growth factors we have tested previously, bFGF has had the greatest effect in promoting hESC self-renewal. Addition of bFGF (also called FGF2) to medium containing a commercially available serum replacement allows the clonal culture of hESCs on fibroblasts[9].

We have previously shown that the addition of BMPs to hESCs cultured in CM containing bFGF promotes trophoblast differentiation[10]. Others have found that blocking BMP activity in serum with the BMP antagonist noggin does not maintain hESC self-renewal, but instead enhances neural differentiation by inhibiting non-neural differentiation[11]. Here we demonstrate that hESCs cultured in UM show greater BMP signaling activity than those cultured in CM, and that this activity is inhibited by the addition of noggin, bFGF or both. We also demonstrate that noggin combined with high bFGF concentrations supports the long-term undifferentiated proliferation of hESCs in the absence of fibroblasts or CM. Three hESC lines, H1, H9 and H14, were used in these studies.

RESULTS

UM contains BMP-like differentiation-inducing activity

UM (see Methods) contained 20% KNOCKOUT serum replacement (Invitrogen), which includes a proprietary lipid-rich bovine albumin component, ALBUMAX[12]. UM was conditioned on fibroblasts overnight and then supplemented with 4 ng/ml human bFGF to obtain CM[13]. We cultured hESCs (H1) in CM, UM, a 1:1 mixture of CM with UM, or a 1:1 mixture of CM with DMEM/F12. The cells in CM or the 1:1 CM-DMEM/F12 mixture remained undifferentiated, and were characterized by typical hESC morphology. However, the cells in UM and in the 1:1 CM-UM mixture both rapidly differentiated within 48 h (**Fig. 1**). We next substituted purified fetal bovine serum albumin (16.6 g/l, Fisher Scientific) for the serum replacement to determine whether albumin caused the differentiation. This medium allowed hESCs to maintain an undifferentiated morphology for about 7 d; however, the cells had a reduced proliferation rate and eventually differentiated into a mixed population of cells (data not shown). These results suggest that components other than albumin contained in the serum replacement are responsible for the rapid differentiation of UM-cultured cells. CM reduces this differentiation-inducing activity, but also provides positive factors to sustain hESC self-renewal. In addition to albumin, serum replacement also contains other components that are required for hESC culture, so serum replacement rather than albumin was used in all subsequent studies.

To examine whether the differentiation-inducing activity in UM stimulates BMP signaling in hESCs, we assessed by western blotting the abundance of phosphorylated Smad1, an immediate effector downstream of BMP receptors[14]. Smad1 phosphorylation (the antibody used here could also detect phosphorylation of the other BMP effectors Smad5 and Smad8; refs. 15 and 16, respectively) was low in H1 cells cultured in CM, but was high in cells cultured for 24 h in UM or in CM plus BMP4 (**Fig. 2a**). The

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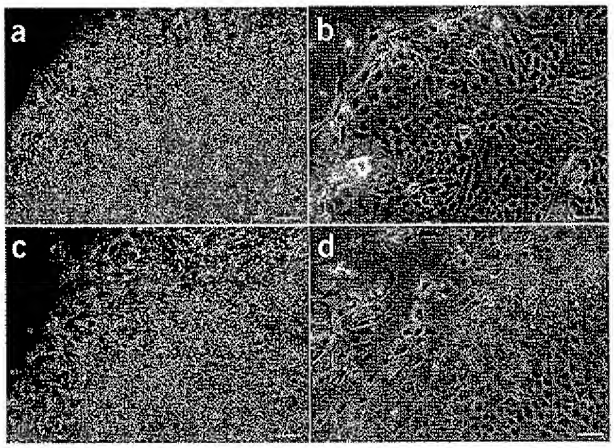

Figure 1 | UM contains differentiation-inducing activity. (**a**–**d**) Photographs of H1 cells cultured in CM (**a**), UM (**b**), CM + DMEM/F12 (1:1) (**c**), and CM + UM (1:1) (**d**) for 7 d. Bar, 50 μm.

addition of noggin to UM reduced Smad1 phosphorylation, but the addition of 40 ng/ml bFGF to UM did not change Smad1 phosphorylation. BMP signaling can induce expression of BMP ligands, forming a positive feedback loop in cells from various species, including hESCs[11]. BMP2 and BMP4 proteins were, indeed, detected at an increased level in UM-cultured hESCs compared to cells cultured in CM or in UM plus noggin (**Fig. 2a**). It is at present unclear whether there are BMPs in UM that directly stimulate BMP signaling in hESCs, or other differentiation-inducing molecules that indirectly stimulate BMP signaling by inducing BMP secretion. Noggin and another BMP antagonist, gremlin[17], were both detected in medium conditioned by the fibroblasts (**Fig. 2b**). These data demonstrate that an elevated, but repressible, BMP signaling activity is present in UM-cultured hESCs, and that both BMP agonists and antagonists are present in fibroblast-supported culture of hESCs.

We further assessed BMP signaling in hESCs (H14) cultured in various media in the presence or absence of protein factors, by using a luciferase reporter plasmid specifically responsive to BMP/Smads[18]. The reporter activity increased with an increasing concentration of the serum replacement or BMP4, and decreased with an increasing concentration of noggin or bFGF (**Fig. 2c**). 500 ng/ml noggin and 40 ng/ml bFGF had a synergistic effect in reducing the reporter activity to the level similar to that achieved by CM. Somewhat surprisingly, even higher amounts of bFGF (100 ng/ml) reduced BMP signaling to a level comparable to that found in CM without the addition of noggin. These results suggest that serum replacement indeed contains BMP-like activity, which can be reduced by noggin and/or bFGF.

Both human *ID1* (ref. 18) and mouse *Id1* (ref. 19) promoters contain BMP-responsive elements[18,19], and *ID1* and *Id1* were previously shown to be targets of BMP4 signaling in both human[10] and mouse[7] ES cells, respectively. We therefore examined the expression of *ID* genes as a second indicator of BMP signaling

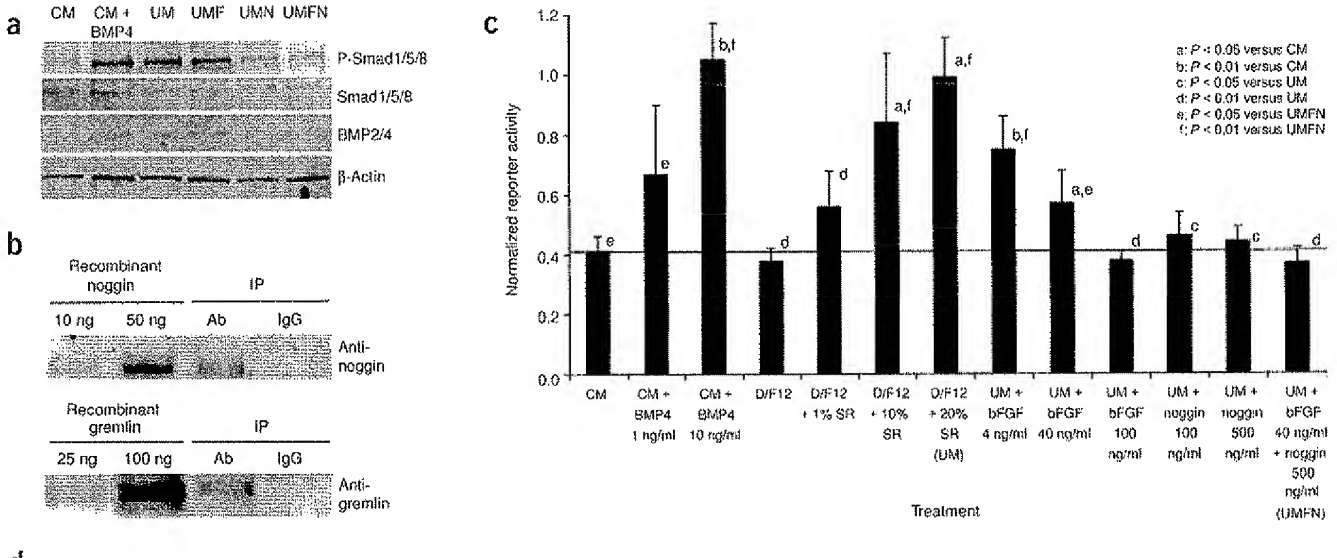

Figure 2 | BMP agonistic and antagonistic signals are detected in hESC culture. (**a**) Western blotting of H1 cells cultured in CM, CM + 100 ng/ml BMP4 (CM +BMP4), UM, UM + 40 ng/ml bFGF (UMF), UM + 0.5 μg/ml noggin (UMN), or UM + bFGF + noggin (UMFN) for 24 h. Phosphorylated Smad1/5/8 (P-Smad1/5/8), Smad1/5/8, BMP2/4 and β-actin (as a loading control) were examined. (**b**) BMP antagonists were detected in serum replacement–free medium conditioned by fibroblasts for 24 h. The media were processed for immunoprecipitation (IP) by goat anti-noggin and anti-gremlin antibodies (Ab) or nonspecific goat immunoglobin (IgG). The precipitated proteins were subjected to western blotting with the anti-noggin and anti-gremlin antibodies separately. Recombinant mouse noggin and gremlin proteins were loaded as positive controls. (**c**) BMP/Smad-luciferase reporter assay on H14 cells cultured for 24 h in CM, DMEM/F12 (D/F12) or UM with or without various concentrations of BMP4, serum replacement (SR), bFGF or noggin. Error bars, ±s.d. (**d**) Quantitative PCR assays for *ID1–4* transcripts in H9 cells cultured in various media for 24 h. Results are displayed as relative mRNA level with the level in CM-cultured cells referred to as 1, and shown as mean ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to CM condition.

activity in hESCs cultured in various media. *ID1–4* transcripts were more abundant in hESCs (H9) cultured for 24 h in UM or CM plus BMP4 than in cells cultured in CM, and addition of noggin to UM reduced expression of the *ID* genes (**Fig. 2d**).

UM + bFGF + noggin sustains hESC self-renewal

UM supplemented with 0.5 μg/ml noggin and 40 ng/ml bFGF sustained undifferentiated proliferation of hESCs (**Fig. 3**). H1 cells were plated at an equal number and cultured for 7 d in CM, UM, UM plus bFGF, UM plus noggin or UM plus bFGF and noggin. $Oct4^+$ cell numbers were significantly higher after 7 d in CM and in UM plus bFGF and noggin than in UM alone, UM plus bFGF or UM plus noggin (**Fig. 3a**). Intermediate $Oct4^+$ cell numbers were detected in UM plus bFGF and UM plus noggin, suggesting a synergistic effect between noggin and bFGF (**Fig. 3a**). hESCs cultured in UM plus bFGF or UM plus noggin could be propagated for multiple passages, but differentiated cells accumulated in either the middle (in UM plus bFGF) or edge (in UM plus noggin) of the hESC colonies (**Supplementary Fig. 1** online). Increased differentiation also occurred in cells cultured in UM plus bFGF and noggin if the noggin concentration was reduced to 0.1 μg/ml and the bFGF concentration to 10 ng/ml (**Supplementary Fig. 1** online). The noggin in UM plus bFGF and noggin could be substituted by gremlin (5 μg/ml) or a soluble BMP receptor IA (0.5 μg/ml) (data not shown), supporting the idea that the effects of noggin are indeed through the interruption of BMP receptor activation by BMPs.

Three hESC lines (H1, H9 and H14) that had been expanded in UM plus bFGF and noggin for more than 40 d (7, 6 and 6 passages, respectively) remained positive for *POU5F1* (also known as *Oct4*), but differentiated if switched to UM lacking bFGF and noggin (**Fig. 3b–d**). hESCs cultured in UM plus bFGF and noggin continued to express other ES cell markers, including *NANOG* and *ZFP42* (an ortholog of mouse *Rex1*; **Fig. 4a**), and the cell surface markers SSEA4 and TRA-1-60 (data not shown). Even in the best cultures, hESCs are mixed with a small percentage of spontaneously differentiated cells. For example, low levels of the trophoblast marker chorionic gonadotropin β-subunit (*CGB*) can be detectable in CM-cultured ES cells, indicating the presence of small populations of trophoblasts[10]. This marker, however, was not detectable in cells cultured in UM plus bFGF and noggin (**Fig. 4a**). Neither the neural progenitor markers *PAX6* and *NEUROD1*; *T*, a homolog of the mouse mesodermal marker *Brachyury*; nor the endodermal marker *FOXA1* (also known as *HNF3α*), were detected in hESCs cultured in CM or UM plus bFGF and noggin (**Fig. 4a**). Thus, ES cells propagated in UM plus bFGF and noggin maintained characteristic ES cell markers following extended culture.

We further examined hESCs after long-term culture in UM plus bFGF and noggin. H9 cells were continuously cultured in UM plus bFGF and noggin for 32 passages. H1 and H14 cells cultured in UM plus bFGF and noggin were frozen after passages 20 and 16, respectively. H14 cells were subsequently thawed directly into UM plus bFGF and noggin and cultured to passage 18. The population doubling time (**Supplementary Table 1** online) and percentage of $Oct4^+$ cells (**Fig. 3a**) of both H9 and H14 cells cultured in UM plus bFGF and noggin for 27 and 18 passages, respectively, were similar to those for CM-cultured control hESCs.

UM + bFGF + noggin maintains hESC developmental potential

When treated with BMP4 in CM for 3–7 d, hESCs that had been previously cultured in UM plus bFGF and noggin for 10 passages differentiated into a flattened epithelium and secreted human chorionic gonadotropin (HCG) into the medium, indicating trophoblast differentiation[10] (**Fig. 4b**). Embryoid bodies derived

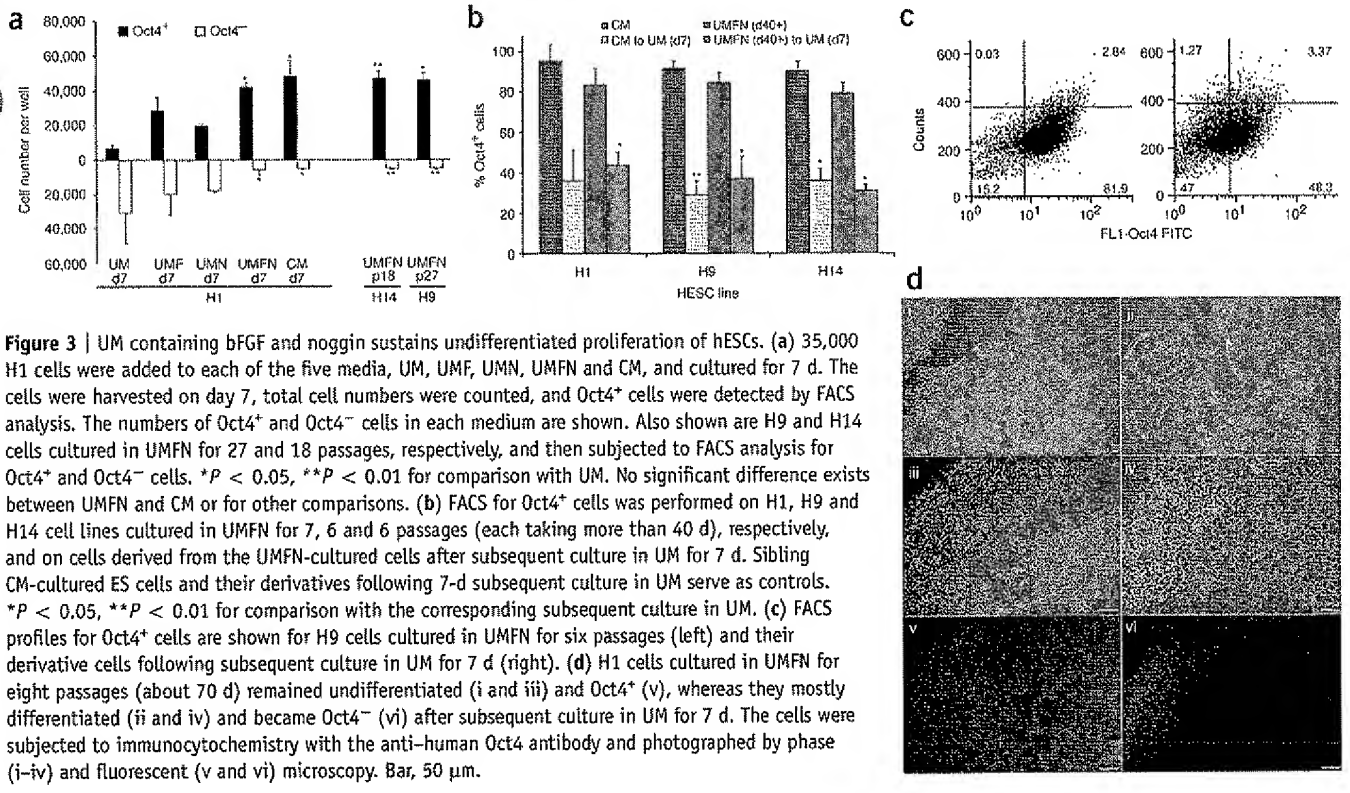

Figure 3 | UM containing bFGF and noggin sustains undifferentiated proliferation of hESCs. (**a**) 35,000 H1 cells were added to each of the five media, UM, UMF, UMN, UMFN and CM, and cultured for 7 d. The cells were harvested on day 7, total cell numbers were counted, and $Oct4^+$ cells were detected by FACS analysis. The numbers of $Oct4^+$ and $Oct4^-$ cells in each medium are shown. Also shown are H9 and H14 cells cultured in UMFN for 27 and 18 passages, respectively, and then subjected to FACS analysis for $Oct4^+$ and $Oct4^-$ cells. $^*P < 0.05$, $^{**}P < 0.01$ for comparison with UM. No significant difference exists between UMFN and CM or for other comparisons. (**b**) FACS for $Oct4^+$ cells was performed on H1, H9 and H14 cell lines cultured in UMFN for 7, 6 and 6 passages (each taking more than 40 d), respectively, and on cells derived from the UMFN-cultured cells after subsequent culture in UM for 7 d. Sibling CM-cultured ES cells and their derivatives following 7-d subsequent culture in UM serve as controls. $^*P < 0.05$, $^{**}P < 0.01$ for comparison with the corresponding subsequent culture in UM. (**c**) FACS profiles for $Oct4^+$ cells are shown for H9 cells cultured in UMFN for six passages (left) and their derivative cells following subsequent culture in UM for 7 d (right). (**d**) H1 cells cultured in UMFN for eight passages (about 70 d) remained undifferentiated (i and iii) and $Oct4^+$ (v), whereas they mostly differentiated (ii and iv) and became $Oct4^-$ (vi) after subsequent culture in UM for 7 d. The cells were subjected to immunocytochemistry with the anti-human Oct4 antibody and photographed by phase (i–iv) and fluorescent (v and vi) microscopy. Bar, 50 μm.

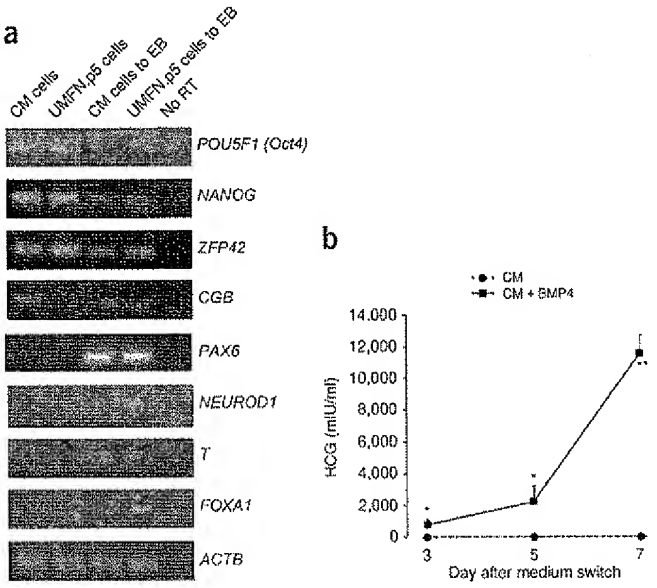

Figure 4 | hESCs cultured in UM containing bFGF and noggin retain developmental potential. (**a**) RT-PCR analysis for molecular markers expressed in H1 cells cultured in CM (CM cells), UMFN for 5 passages (UMFN.p5 cells), and differentiated cells through embryoid body formation from these two groups of cells, respectively (CM cells to EB and UMFN.p5 cells to EB). The 'UMFN.p5 cells to EB' sample, processed for RT-PCR without reverse transcriptase (RT), served as a negative control (NO RT). (**b**) HCG secreted by UMFN-cultured hESCs in response to BMP4. H9 cells cultured in UMFN for 10 passages and then subcultured in CM or CM + 100 ng/ml BMP4 for 7 d with daily refreshment of the medium and BMP4. Spent media were collected from the subcultures on days 3, 5 and 7 after the medium switch and tested for HCG. $^{*}P < 0.05$, $^{**}P < 0.01$ between HCG level in the CM + BMP4 culture and that in the CM culture.

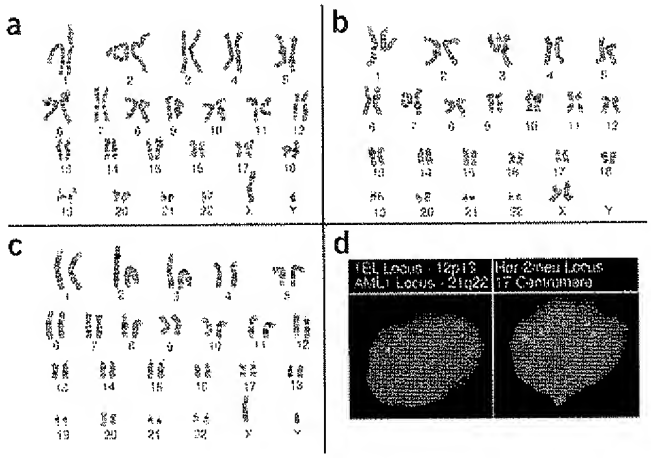

Figure 5 | hESCs cultured in UM containing bFGF and noggin remain karyotypically normal. (**a–c**) H1 cells cultured in UMFN for 5 passages (**a**), H9 for 32 passages (**b**) and H14 for 19 passages (**c**) were karyotyped by standard G banding. (**d**) The diploidy of chromosomes 12, 17 and 21 in the UMFN-cultured cells was confirmed via detection of marker genes for these chromosomes by fluorescence *in situ* hybridization.

from H1 cells cultured in UM plus bFGF and noggin for 5 passages, and from control CM-cultured cells, expressed the trophoblast marker *CGB* and markers of the three germ layers, including *PAX6*, *NEUROD1*, *T* and *FOXA1*. Embryoid body cells also had reduced expression of the ES cell markers *Oct4*, *NANOG* and *ZFP42* (**Fig. 4a**). H1 and H9 cells cultured in UM plus bFGF and noggin for 7 and 6 passages, respectively, were injected into SCID-beige mice. Teratomas exhibiting complex differentiation developed in the mice 5–6 weeks after inoculation (**Supplementary Fig. 2** online).

ES cells in UM + bFGF + noggin have normal karyotype

H1 cells cultured in UM plus bFGF and noggin for 5 passages, H9 for 33 passages and H14 for 19 passages were karyotyped by standard G-banding, and chromosomes 12 and 17 (ref. 20) were examined by fluorescence *in situ* hybridization. The cells retained normal karyotypes (**Fig. 5**).

DISCUSSION

The evolutionary conservation of important developmental pathways across divergent model organisms is a central concept in modern developmental biology. There is, therefore, a high expectation that mechanisms controlling self-renewal and pluripotency in various mammalian ES cells should share essential features. Recent expression analyses of human and mouse ES cells confirm that there is a substantial overlap in the expression of key genes[21–24], and this overlap has already been important in directing studies of self-renewal. For example, elements of the TGFβ and Wnt signaling pathways are well represented in both mouse and human ES cells[21–24], which led to the testing of TGFβ1 (ref. 25) and Wnt3a (ref. 26) on hESC self-renewal.

Yet there appear to be important differences in the growth factor requirements of mouse and human ES cells. BMP signaling through *Id* genes and LIF signaling through *Stat3* are sufficient to promote the clonal growth of mouse ES cells in serum-free medium[7]. Activation of the LIF/Stat3 signaling pathway[1,8] does not sustain hESCs in conditions that would support mouse ES cells, and we have been unable to culture undifferentiated hESCs in serum-free medium supplemented only with LIF and BMP4 (unpublished data). BMPs cause hESC differentiation either to trophoblast[10] or primitive endoderm[11] in conditions that otherwise support their undifferentiated proliferation. The BMP/Smad reporter assay indicated the presence of BMP-like activity in UM (containing 20% serum replacement) that was roughly comparable to that in CM supplemented with 10 ng/ml BMP4 (**Fig. 2c**), a concentration we have previously shown causes differentiation[10]. There remain low but detectable levels of Smad1 phosphorylation and BMP2 and BMP4 production in current conditions that maintain undifferentiated hESC proliferation, including culture on fibroblasts[11], in CM and in UM plus noggin and bFGF (**Fig. 2a**). Although it is possible that this low level of Smad1 phosphorylation is from contaminating differentiated cells rather than from the hESCs themselves, a role for BMP signaling in hESC self-renewal cannot be completely ruled out yet. In every condition we have tested to date, however, suppression of BMP signaling has been beneficial and not deleterious to hESC culture.

bFGF allows the clonal growth of hESCs on fibroblasts in medium containing serum replacement[9], and the same medium supports hESCs after conditioning on fibroblasts[13]. Both the fibroblasts (unpublished data) and hESCs express multiple FGF genes, and ES cells themselves express multiple FGF receptors[22]. The increased concentration of bFGF (40 ng/ml) used in the

current studies allowed hESCs to be expanded through multiple passages even in unconditioned medium without noggin. During the first several days after plating, cultures in UM plus bFGF and in UM plus bFGF and noggin seemed similar, but the proportion of differentiated cells consistently increased, especially in the middle of the cell colonies, with each culture passage in UM plus bFGF. By contrast, in the UM cultures containing bFGF and noggin, the differentiated cells were largely eliminated (**Supplementary Fig. 1** online). Although 500 ng/ml noggin (a median effective dose (ED_{50}) recommended by the manufacturer) was used throughout this study, we found that 100 ng/ml noggin was also sufficient to demonstrate the same effect as 500 ng/ml when used together with 40 ng/ml bFGF (data not shown). The noggin doses we used were far higher than the detected level in CM (**Fig. 2b**), so it is unlikely that noggin alone is the cause of reduced BMP signaling activity in CM-cultured cells.

Although bFGF did not seem to inhibit Smad1 phosphorylation (**Fig. 2a**), the BMP/Smad reporter assay indicated that downstream BMP signaling events were inhibited by bFGF (**Fig. 2c**). Previous studies have shown an ability of bFGF to interrupt BMP signaling either by preventing the nuclear translocation of phosphorylated Smad1 (ref. 27) or by repressing Smad1 activity in the nucleus[28]. Notably, at 100 ng/ml bFGF, BMP signaling activity was repressed to levels observed in CM, even without the addition of noggin (**Fig. 2c**). Indeed, we have found recently that at 100 ng/ml bFGF, an effect of noggin can no longer be demonstrated, and hESCs can be maintained through multiple passages with minimal background differentiation (unpublished data). Clearly, other effects of these high concentrations of bFGF are also important to hESC self-renewal, yet suppressed BMP signaling remains a consistent hallmark of current methods of hESC culture.

Undifferentiated hESCs can be sustainably expanded in the described culture system, yet it is important to examine the limitations and the challenges that remain. First, although the elimination of fibroblasts is an important step in the development of a defined medium, UM containing bFGF and noggin is not yet a defined medium. Both the serum replacement and the cell matrix Matrigel, which was used to coat plates for hESC culture, are complex mixtures of animal origin. Although we have had some success replacing Matrigel with laminin, we have observed marked differences in effectiveness between different commercial sources of both mouse and human laminins, and even between batches of laminin from the same source. Second, UM containing bFGF and noggin supports the clonal growth of hESCs extremely poorly, if at all. Cells need to be plated in clumps, and in general, higher-density cultures grow better. Finally, although this medium did support each of the hESC lines we tested, the true test of a new medium will be whether it supports growth from initial derivation. In spite of these limitations, our results define some of the signals important in the self-renewal of hESCs, eliminate the need for some animal-derived materials, and should further facilitate the use of hESCs as an experimental model.

METHODS

Medium and cell culture. UM contained 80% DMEM/F12 and 20% KNOCKOUT serum replacement, and was supplemented with 1 mM L-glutamine, 1% nonessential amino acids (all from Invitrogen) and 0.1 mM 2-mercaptoethanol (Sigma). CM was prepared as described[13]. hESCs were cultured on plates coated with Matrigel (BD Scientific) in CM or UM with or without either 0.5 μg/ml mouse noggin (R&D Systems), or 40 ng/ml human bFGF (Invitrogen), or both, and propagated by using 2 mg/ml dispase (Invitrogen) to loosen the cell colonies. For evaluation of $Oct4^+$ cell number, suspended colonies containing 35,000 cells were added to each medium in multiple wells and cultured for 7 d. Cells were harvested and counted on days 1 and 7, and $Oct4^+$ cells on day 7 were detected by fluorescence-activated cell sorting (FACS, see below). Embryoid bodies were formed by suspending hESCs that had been cultured in CM or in UM containing bFGF and noggin (UMFN) as cell clumps in UM on a noncoated plate and culturing them on a rocker for 7 d. The embryoid body cells were then replated in DMEM medium supplemented with 10% fetal bovine serum on a gelatin-coated plate and cultured for 5 d followed by harvesting and RT-PCR analysis. Experiments were repeated multiple times and ANOVA was used for statistic analysis throughout the studies.

Immunoprecipitation and western blotting. 15 ml of DMEM/F12 medium was conditioned by 3.8×10^6 irradiated mouse embryonic fibroblast cells in a T75 flask overnight. The medium was collected and concentrated to about 0.7 ml with a 5 kDa molecular weight cutoff filter (Millipore) and immunoprecipitated with goat anti-mouse noggin and gremlin antibodies (R&D Systems; 5 μg each) or 10 μg goat IgG as a negative control. The precipitated proteins (**Fig. 2b**) or cell lysates (**Fig. 2a**) were electrophoresed on a 4%–20% linear gradient polyacrylamide Tris-HCl precast gel (Bio-Rad) for western blotting. The antibodies against mouse noggin and gremlin were used for the immunoprecipitated proteins, and antibodies against human Smad1/5/8, phosphorylated Smad1/5/8 (Cell Signaling Technology), BMP2/4 (R&D Systems) and β-actin (Abcam) were used for the cell lysates. The blots were treated with the ECL substrate solutions 1 and 2 (Amersham Biosciences) and exposed in a Fuji Imager (Fuji Medical Systems) for chemiluminescence.

BMP/Smad luciferase reporter assay. hESCs cultured in CM were transfected with a BMP/Smad-responsive firefly luciferase reporter plasmid, pID120-Lux[18], together with trace amount of pRL-tk plasmid (Promega) to express Renilla luciferase as an internal control. One day after transfection, the cells were treated variously for 24 h. Cell lysates were extracted and both the firefly and Renilla luciferase activities tested by using the Dual-Luciferase Reporter Assay System (Promega) on a 3010 Luminometer (BD Biosciences). Results are shown as the firefly luciferase activity normalized to the Renilla luciferase activity.

Quantitative PCR and RT-PCR. Total cellular RNA was extracted using a RNeasy kit (Qiagen) and treated with RNase-free DNase according to the manufacturer's instructions. Then, 1 μg RNA was reverse transcribed to cDNA with Improm-II Reverse Transcription System (Promega). Quantitative PCR was performed using the SYBR green Q-PCR mastermix (Stratagene) on the AB 7500 Real Time PCR System (Applied Biosystems) under the following conditions: 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C; and 3 min extension at 72 °C. *GAPDH* transcript was tested as an endogenous reference to calculate the relative expression levels of target genes according to Applied Biosystems' instructions. For RT-PCR, the following conditions

were used: 3 min at 94 °C, and then various cycles (see below) of 20 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C. The PCR reactions were separated by gel electrophoresis and the DNA bands were visualized under ultraviolet light for photography. The primer sequences and PCR cycle numbers are listed in **Supplementary Table 2** online.

FACS and immunocytochemistry. hESCs cultured in various media were processed for FACS analysis[10] to detect Oct4$^+$ cells. Mouse anti-human Oct4 antibody (Santa Cruz Biotechnology) at 2 μg/ml and fluorescent isothiocyanate–labeled rabbit anti-mouse secondary antibody (Molecular Probes) at 1:1,000 dilution were used. Statistical analysis was performed on Arcsine values converted from the percentages of Oct4$^+$ cells. For immunocytochemistry[10], the mouse anti-Oct4 (at 2 μg/ml) antibody was used and followed by Alexa Fluor 488-labeled anti-mouse IgG secondary antibody (Molecular Probes) at 1:1,000 dilution.

Immunoassay of HCG in the culture medium. hESCs cultured in UMFN for multiple passages were subsequently cultured in CM plus 100 ng/ml BMP4 up to 7 d with daily refreshment of the medium and BMP4. The spent media were collected on days 3, 5 and 7, and assayed for HCG as described[10].

G-banding and fluorescence *in situ* hybridization. hESCs cultured in UMFN for various passages were processed for G-banding and fluorescence *in situ* hybridization as described[20,29,30]. From all the dispersed and fixed cells, 20 cells at metaphase were analyzed for G-banding, and 100–200 nuclei were assayed for fluorescence *in situ* hybridization using probes to detect marker genes in chromosomes of interest. Representative images captured by the CytoVysion digital imaging system (Applied Imaging) are shown.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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